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Expression of human blood clotting factor VIII in the mammary gland of transgenic sheep

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Abstract

By targeting the expression of sequences encoding non-milk proteins to the mammary gland of transgenic farm animals, the organ could serve as a 'bioreactor' for producing pharmacologically active proteins on a large scale. Here we report the generation of transgenic sheep bearing a fusion gene construct with the human blood clotting factor VIII (hFVIII) cDNA under the transcriptional control of a 2.2 kb fragment of the mammary gland speci c promoter of the ovine ß-Lactoglobulin (ß-Lac) gene. Six founder animals were generated bearing a hFVIII cDNA construct with the introns of the murine metallothionein (MtI) gene (ß-Lac/hFVIII-MtI). Founders transmitted the transgene in a Mendelian fashion and two transgenic lines were generated. Ten out of 12 transgenic F1-females expressed rhFVIII mRNA in exfoliated mammary epithelial cells isolated from the milk. But only in transgenic F1 ewes 4010 and 603 hFVIII clotting activity estimated at 4–6 ng/ml was detected in defatted milk. Furthermore, the presence of rhFVIII-protein in ovine milk was demonstrated by a speci c band at approximately 190 kD following immunoprecipitation and immunoblotting. Transgenic founder 395 expressed rhFVIII mRNA in biopsied mammary gland tissue, in exfoliated mammary cells as well as ectopically in brain, heart, spleen, kidney and salivary gland, suggesting that the employed ß-Lac promoter fragment lacks essential sequences for directing expression exclusively to the mammary gland. A rhFVIII standard preparation ($rhFVIII^{std}$) was rapidly sequestered in a saturable fashion in ovine milk, thus rendering it largely inaccessible to immunoprecipitation although its biological activity was retained. Recovery of $hFVIII^{std}$ was dependent on milk donor, storage temperature and dilution of milk sample.

Introduction

Human antihemophilic factor VIII (hFVIII) is a plasma glycoprotein that plays a crucial role in blood coagulation (Fallaux et al., 1995). The hFVIII gene has a size of 186 kb, consists of 26 exons and 25 introns and encodes a 9 kb mRNA (Gitschier et al., 1984; Toole et al., 1984; Vehar et al., 1984; Wood et al., 1984). The hFVIII-polypeptide is synthesized predominantly in hepatocytes as a single chain macromolecule and in human blood hFVIII is closely associated with von Willebrand factor (vWF) (Wood et al., 1984). Congenital X-linked de ciency of hFVIII (hemophilia A) is the most common human bleeding disorder affecting approximately one of 5000 males (Tuddenham et al., 1991). Treatment of hemophilia A involves complementation of patients with either hFVIII concentrates derived from blood of healthy individuals or with recombinant hFVIII preparations, which have become available recently

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(Kaufman, 1989; Rosendaal et al., 1991; Fallaux et al., 1995). However, the supply of conventional or rhFVIII preparations is by far insuf cient to cover the worldwide demand (Soutar, 1994) and risks of contamination and antigenic responses remain.

An alternative approach to alleviate the above problems could be the use of transgenic farm animals bearing appropriate gene constructs directing the expression of sequences encoding hFVIII into the mammary gland. Following hFVIII secretion into milk, a pharmaceutical product could be readily isolated and puri ed (Clark et al., 1987; Hennighausen et al., 1990). Work in several laboratories has indicated that cDNA-containing gene constructs are frequently poorly expressed in transgenic animals, and that transgene expression could be rescued by introducing either homologous or heterologous intron sequences into the cDNA (Brinster et al., 1988; Choi et al., 1991; Palmiter et al., 1991). Recent work in our laboratory has demonstrated that the fusion gene ß-Lac/hFVIII-MtI, in which the hFVIII cDNA has been ligated to the 5′ end of the coding region of the murine metallothionein I gene under the transcriptional control of a 2.2 kb fragment of the ß-Lac promoter, was expressed in the mammary gland of transgenic mice (Espanion et al., 1997). Here we describe the generation of transgenic sheep expressing the fusion gene construct ß-Lac/hFVIII-MtI in the mammary gland and the presence of rhFVIII protein and clotting activity in the milk of transgenic ewes.

Material and methods

Cloning procedures and generation of transgenic sheep

The cloning of ß-Lac/hFVIII gene constructs with or without the mürine Mt-I-gene has been described previously (Halter et al., 1993). We used the construct ß-Lac/hFVIII-MtI for these experiments. For microinjection, linearized DNA was used without vector sequences. During three consecutive breeding seasons transgenic sheep were generated via microinjection into pronuclei of zygotes recovered from superovulated ewes and identi ed by Southern analyses of DNA samples obtained from tail biopsies (Halter et al., 1993; Guzik and Niemann, 1995).

RNA analysis

Mammary gland tissue biopsies were obtained from lactating transgenic female 395. Exfoliated mammary epithelial cells were collected from the milk of lactating transgenic ewes by centrifugation for 10 min and 365 × *g* at 4°C. The resulting pellet was stored frozen at −80°C. Tissue samples from various organs were taken from transgenic female 395, which had to be euthanized, to analyse ectopic transgene expression. Total RNA from the cells or tissues was prepared, using the guanidinium-thiocyanate-phenol-chloroform extraction procedure (Chomczyinski and Sacchi, 1987). From exfoliated mammary epithelial cells isolated from ovine milk by centrifugation, total RNA was prepared by employing the Qiagen(r)-RNeasy total RNA kit. Northern blotting failed to demonstrate transgene-encoded mRNA expression. The detection of hFVIII mRNA was carried out by reverse transcriptase PCR (RT-PCR), employing 35 PCR cycles (60.4°C: primer RH3/RH4 annealing; 72°C: extension; 94°C: denaturation). The primers RH3 (5′-GCCTCTCAGAGTCACCACTTCCTCTGTTGT-3′) and RH4 (5′- AAGACGCTGGGTTG-GTCCGATAC TATTTAC-3′) were used to amplify a 334 bp fragment from the rhFVIII mRNA. The PCR fragment consists in its 5′ portion of hFVIII cDNA encoded sequences (80 bp), and in its 3′ portion of MtI sequences following splicing of introns 1 and 2 (254 bp). The identity of the RT-PCR fragment was ensured by a SmaI digest and sequencing. RNA analysis from tissues of non-transgenic animals never revealed a speci c signal.

Detection of hFVIII in ovine milk

Milk samples were collected 8–12 days after onset of lactation when the colostrum phase was terminated. Approximately 50 ml milk was collected three times per week by hand-milking after the lamb had been separated from the mother for approximately 2–3 h. Milk samples were collected over a period of 6–7 weeks until lactation ceased. Milk was stored frozen at −80°C until analysis.

Clotting activity in milk

The principle of this bioassay is based on the commercially available Immunochrom FVIII: C blood Reagent Kit (Immuno, Heidelberg, Germany) allowing chromogenic determination of hFVIII activity. This test system usually serves for routinely diagnosing aberrant FVIII-levels in human plasma. The change in the colour of the chromogenic substrate is determined photometrically. According to the manufacturers' information 1% hFVIII-activity corresponds to 1 IU/ml

FVIII (WHO). This assay permits a direct measurement of hFVIII activity. Non-transgenic milk did not produce a signal in this assay. $CaCl_2$ was added to 1 ml milk samples to a nal concentration of 250 mM, followed by incubation at room temperature for 10 min and centrifugation at 4°C for 30 min. The supernatant without fat was centrifuged again and used for the test in a 1:5 dilution in dilution buffer (Immuno). As a positive control, rhFVIII^{std} at different concentrations incubated in non-transgenic milk was used. Milk samples from non-transgenic sheep were prepared in the same manner and used as negative controls. The clotting assay was shown to permit determinations of rhFVIII^{std} at levels >1 ng/ml.

Immunoprecipitation and immunoblotting

Milk samples were diluted 1:1 in 2× RIPA buffer (Santa Cruz) and incubated for 10 min at room temperature, followed by centrifugation at 4°C for 30 min. The supernatant without fat was centrifuged again. The supernatant (1500 µl) was immunoprecipitated by rotation at 4°C overnight by employing 50 µl of a mix consisting of 10 µl protein AG linked to agarose beads (Santa Cruz) and 4 µl goat anti-hFVIII antiserum, preincubated for 1 h in a nal volume of 50 µl RIPA buffer. The immunoprecipitate was washed 3× in RIPA buffer, boiled for 2 min in sample buffer and loaded onto 6% SDS-PAGE (Laemmli, 1970). After blotting onto nitrocellulose (Schleicher and Schuell, Germany), rhFVIII^{std} speci c bands were detected by using a monoclonal antibody against the C-domain of hFVIII (Calbiochem 233413, anti hFVIII-c) and with speci c peroxidase labelled anti mouse IgG Fab-fragments (Dianova 305-036-045) by employing the ECL detection system (Amersham). The rhFVIII^{std}was visualized by two bands of 80 and 73 kD showing the normally processed fragments from the hFVIII precursor. Western blot analysis and quantitative densitometry determined levels of rhFVIII^{std} in ovine milk.

For identi cation of rhFVIII in the milk of transgenic ewes, 6 ml milk from transgenic and non-transgenic sheep were diluted 1:1 in 2× RIPA buffer and prepared as described before. For immunoprecipitation of rhFVIII, 20 µl of a mix (2 µl goat anti-hFVIII antiserum, 4 µl protein A/G coupled to agarose beads (Santa Cruz) in RIPA buffer) were added to 1500 µl supernatant of the milk preparation (for each ovine milk sample 6 aliquots of 1500 µl each were produced) and incubated by rotation at 4°C overnight. The immunoprecipitated aliquots from each milk sample were pooled and collected in two fractions, washed as described above and boiled for 2 min in sample buffer. Both fractions of each milk sample were loaded onto a 6% denaturating SDS-PAGE (Laemmli, 1970). After electroblotting onto nitrocellulose (Schleicher and Schuell) rhFVIII speci c bands were detected as described above. As a positive control, 60 ng of rhFVIII^{std} in 1500 µl of RIPA (0.025% BSA) was immunoprecipitated with 20, 60 and 120 µl of the mix used for immunoprecipitation. It was observed that 60 µl were suf cient for 100% immunoprecipitation of 60 ng rhFVIII (Figure 1, lanes 7–9).

Results

Generation of transgenic sheep

A total of 277 microinjected zygotes were transferred to 106 recipients, yielding 69 (65.1%) pregnancies and 103 (37.2%) born lambs from which six transgenic founder sheep (2.2% of lambs born) were identi ed. The two transgenic males (393 and 394) transmitted the transgene in a Mendelian fashion and each one produced seven female transgenic ewes, from which 12 animals (including F1 ewes 4010 and 603) were analyzed for expression of rhFVIII in milk during lactation. The transgenic founder ewe 395, from which biopsies were taken from its mammary gland during the lactation phase following the rst parturition, had to be euthanized following premature delivery of twins in the second parity and was therefore analyzed for ectopic transgene expression. Transgenic founder 436 died at 2 years of age without producing offspring while a pair of transgenic twins was born dead.

Expression of hFVIII constructs in transgenic sheep

In biopsied mammary gland tissue from transgenic founder 395, hFVIII mRNA was unequivocally identi ed by the presence of the predicted 334 bp RT-PCR fragment, which was veri ed by the predicted fragments of 80 and 254 bp, resulting from restriction analysis and sequencing showing 100% homology with the sequence in the database. In addition, the presence of rhFVIII mRNA was demonstrated in exfoliated mammary epithelial cells isolated from the milk during various phases of lactation following the rst parturition. Even though low (1 ng/ml) levels of rhFVIII clotting activity were observed in the milk

during different phases of lactation, clear and consistent evidence for the presence of rhFVIII in milk could not be obtained. The transgene was ectopically expressed in brain, heart, liver, spleen, kidney and salivary gland, however, not in lung and muscle.

Ten out of the 12 transgenic F1-females expressed rhFVIII-mRNA in exfoliated mammary epithelial cells isolated from milk. However, rhFVIII was only detected in the milk of two animals. During lactation of transgenic ewes 4010 (F1 from transgenic male founder 393) and 603 (F1 from transgenic male founder 394) rhFVIII mRNA was expressed in exfoliated mammary epithelial cells isolated from milk (Figure 1a). To identify rhFVIII in milk, clotting assays were performed after the secretion of colostrum had ceased (day 7 after onset of lactation). As shown in Figure 1b (for 4010) rhFVIII clotting activity was clearly and consistently (at least 4 repeats of each analysis) present in milk starting at day 12, and increased continuously until the end of lactation (day 42). Similar findings were made for transgenic ewe 603 (data not shown).

Immunoprecipitation of rhFVIII in milk samples of transgenic ewe 4010 showed a specific band of about 190 kD (Figure 1c, lane 3). This band was never observed in negative controls. This size did not correspond to the expected two fragments of 80 and 73 kD, that result from normal processing of the 200 kD precursor. The presence of a 190 kD rhFVIII fragment indicates that proteolytic processing of the rhFVIII precursor is achieved by a different mechanism in sheep milk. In fact, a 190 kD fragment is expected if the initial proteolytic step occurred at position 740/741 instead of 1648/1649 (Pittman and Kaufman, 1989). It consists of the B domain and the A_3, C_1 and C_2 domains. This fragment which includes the C domain was visualized after immunoblotting with a hFVIII C domain specific MAB. Control experiments showed that the conditions for immunoprecipitation were appropriate for a quantitative precipitation of rhFVIII from milk of transgenic ewe 4010. Consequently a constant amount of $rhFVIII^{std}$ was immunoprecipitated with different amounts of rhFVIII in milk (Figure 1c). These experiments conclusively confirmed that the amount of antibody was sufficient for the immunoprecipitation of 60 ng/$rhFVIII^{std}$ and could therefore safely be employed for analysis of ovine milk in which rhFVIII activity had been detected at concentrations of about 4–6 ng/ml.

The amounts of detectable rhFVIII in the milk of transgenic ewe 4010 differed between the two test

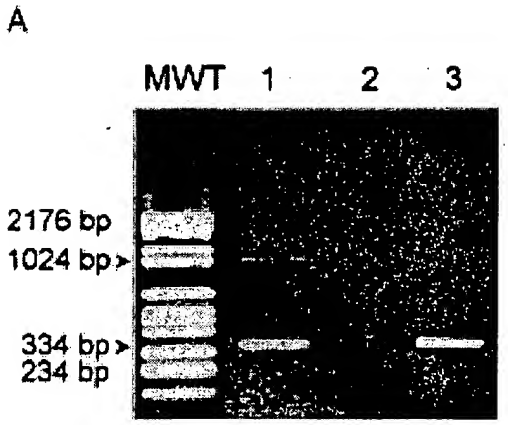

Figure 1a. RT-PCR analysis of 2 µg total RNA from exfoliated epithelial cells isolated from milk of transgenic sheep 4010. RT-PCR products generated from exfoliated mammary epithelial cells from sheep 4010 (lane 1) and as a negative control from a non-transgenic sheep (lane 2). As a positive control, total RNA from the mammary gland of a transgenic mouse expressing hFVIII mRNA was used (lane 3). The RT-PCR product of 1024 bp indicates DNA contamination of the total RNA (lane 1).

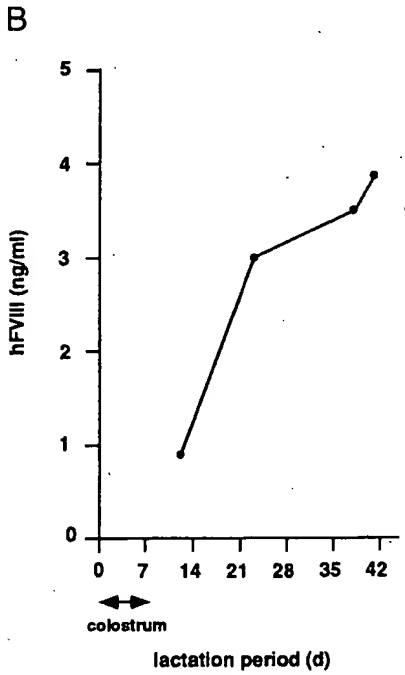

Figure 1b. Clotting activity of rhFVIII in milk of transgenic sheep 4010. Milk samples collected at the indicated times following onset of lactation and after the secretion of colostrum ceased at day 7, were assayed for hFVIII activity by employing the clotting assay (see Experimental Protocol for details). In a control experiment $rhFVIII^{std}$ was used as a standard to estimate rhFVIII concentration in the milk in ng/ml.

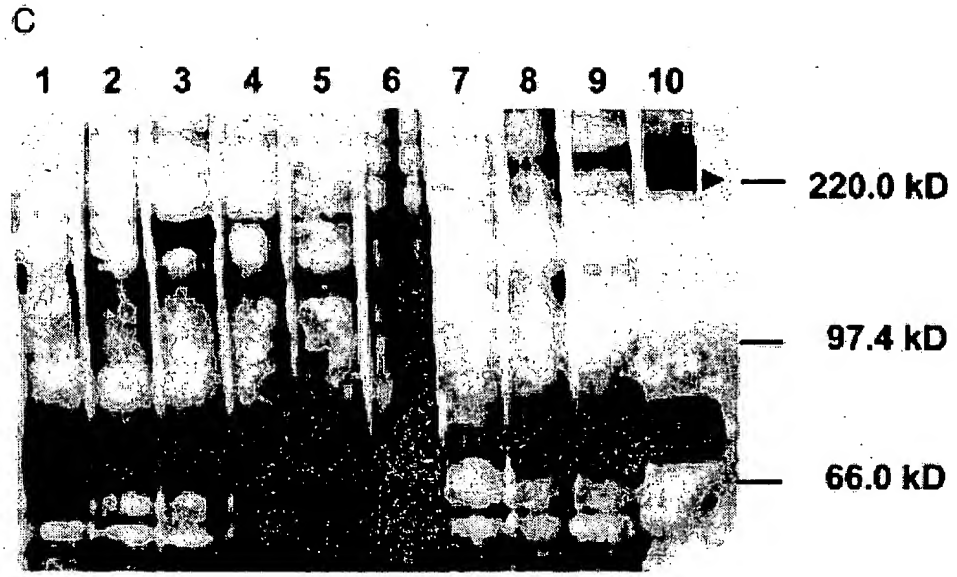

Figure 1c. Identi cation of rhFVIII in the milk of transgenic ewe 4010. For immunoblotting 6 ml of milk from transgenic and non-transgenic sheep were analyzed for the presence of rhFVIII speci c bands. Lanes 1, 2 and 6: three non-transgenic ewes; lane 3: transgenic ewe 4010; note the speci c band at 190 kD; lanes 4 and 5: transgenic ewe 509 in which no rhFVIII activity was detected; lanes 7–9: immunoprecipitation controls: 60 ng rhFVIII^{std} using various amounts of immunoprecipitation mix (lane 7: 20 µl; lane 8: 60 µl and lane 9: 120 µl) per lane; lane 10: direct immunoprecipitation of rhFVIII. To minimize the background for immunoprecipitation we used fewer protein A/G-agarose beads per ml (3 µl/ml) as performed above (20 µl/ml).

systems employed. In an effort to better understand these discrepancies as well as the reasons for the low rhFVIII concentrations, we have completed a series of experiments employing in parallel immunoprecipitation and the activity test, using the rhFVIII^{std} preparation as a model for the endogenous rhFVIII.

Recovery and stability of rhFVIII^{std} in non-transgenic ovine milk

Detection of rhFVIII^{std} by immunoprecipitation/immunoblotting failed at levels below 100 ng/ml in non-transgenic ovine milk. Addition of 2× RIPA buffer to milk led to increased rhFVIII^{std} recovery by immunoprecipitation/immunoblotting. Detection of rhFVIII^{std} activity in untreated ovine milk by the clotting assay failed at levels below 7 ng/ml, whereas in the presence of $CaCl_2$ (250 mM) the limit for the detection of rhFVIII^{std} was >1 ng/ml. This observation suggested that rhFVIII^{std}was at least in part sequestered by milk components and that the addition of $CaCl_2$ caused the partial release of cryptic rhFVIII^{std}. In blood hFVIII is non-covalently bound to vWF; $CaCl_2$ was used to separate both components from each other. However, addition of vWF in the presence or absence of $CaCl_2$ did not affect the accessibility of rhFVIII^{std} to immunoprecipitation (data not shown). Furthermore, the recovery by immunoprecipitation/immunoblotting of exogenously added rhFVIII^{std} following incubation in milk varied signi cantly among milk samples collected from three non-transgenic sheep after processing in RIPA buffer (data not shown).

Recovery rates of rhFVIII^{std} from ovine milk by immunoprecipitation/immunoblotting and clotting assays are dependent on levels added, dilution of milk samples and temperature

rhFVIII^{std} was added at levels of 10 or 60 ng/ml to diluted milk samples obtained from a non-transgenic sheep, incubated for 5 h at 30°C and recovery of rhFVIII^{std} was analysed by immunoprecipitation/immunoblotting. The results showed that recovery rates of rhFVIII^{std} depended on the concentration of rhFVIII^{std} as well as on the extent to which milk samples had been diluted. Recovery rates of rhFVIII^{std} were high following incubation in TBS buffer only or in highly diluted milk samples. However, low or

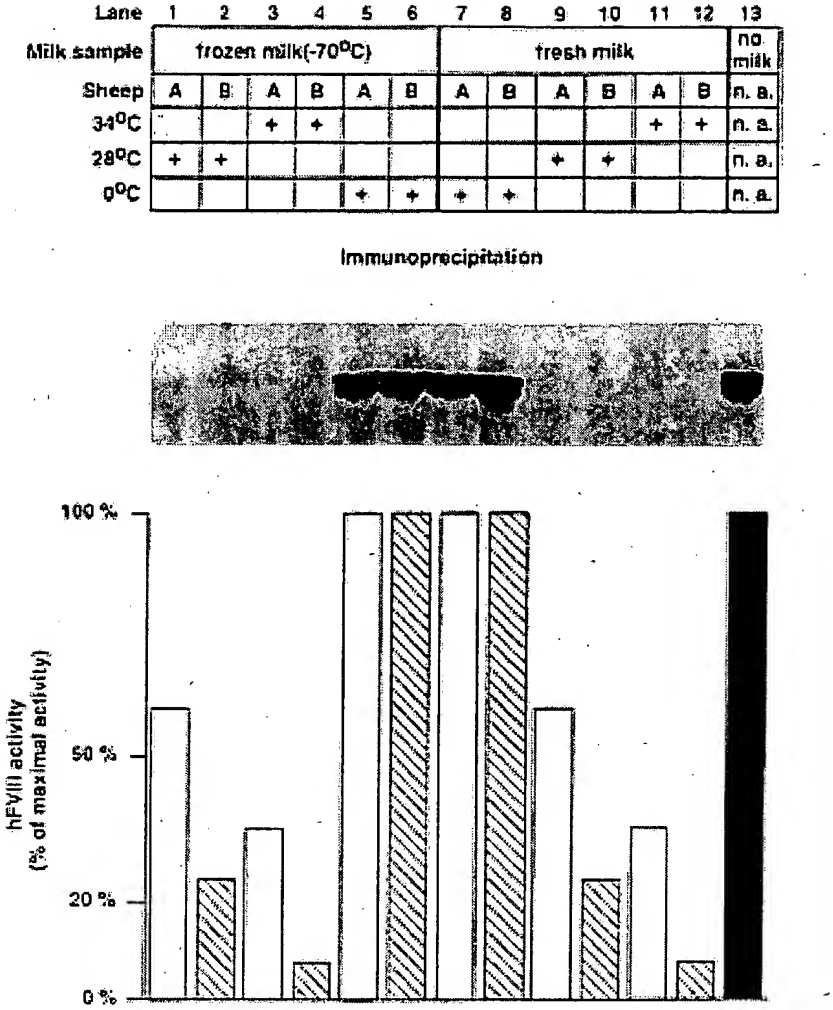

Figure 2a. Recovery of rhFVIII^{std} (120 ng/ml) in the immunoprecipitation/ immunoblotting assay (center panel) and in the biological (clotting) assay (low panel) following incubation in 1 ml milk samples of two non-transgenic sheep A and B (top panel), sheep A (open columns), and sheep B (hatched columns). Undiluted milk was used either after thawing (frozen (−70°C) milk samples: lanes 1–6) or immediately after collection (lanes 7–12). Samples were incubated at 0, 28 or 34°C for 20 h as indicated in the top panel. Following incubation, the samples were split and aliquots of 600 µl or 400 µl allocated for immunoprecipitation/immunoblotting assays or biological assays, respectively. Recovered rhFVIII^{std} in the immunoprecipitation/immunoblotting assay (center panel: lanes 1, 3, 5, 7, 9, 11 for sheep A, and in lanes 2, 4, 6, 8, 10, 12 for sheep B), and in the biological assay (low panel). Controls: Recovered rhFVIII^{std} from RIPA buffer without previous incubation in milk in the immunoprecipitation/immunoblotting assay (center panel, lane 13), and in the biological assay (low panel, lane 13 lled column).

virtually no rhFVIII^{std} was recovered from samples containing the highest milk proportion.

Following incubation of rhFVIII^{std} (150 ng/ml) in milk from a non-transgenic sheep for either 0, 6 or 20 h at 30°C, signi cant losses were observed with increasing duration of incubation (data not shown).

Thus, it appeared that rhFVIII^{std} is sequestered by milk components in a concentration-dependent and saturable fashion, rendering rhFVIII^{std} in part cryptic to immunoprecipitation. Proteolytic activity in the milk leading to rhFVIII^{std} degradation was demonstrated by the presence of breakdown products as

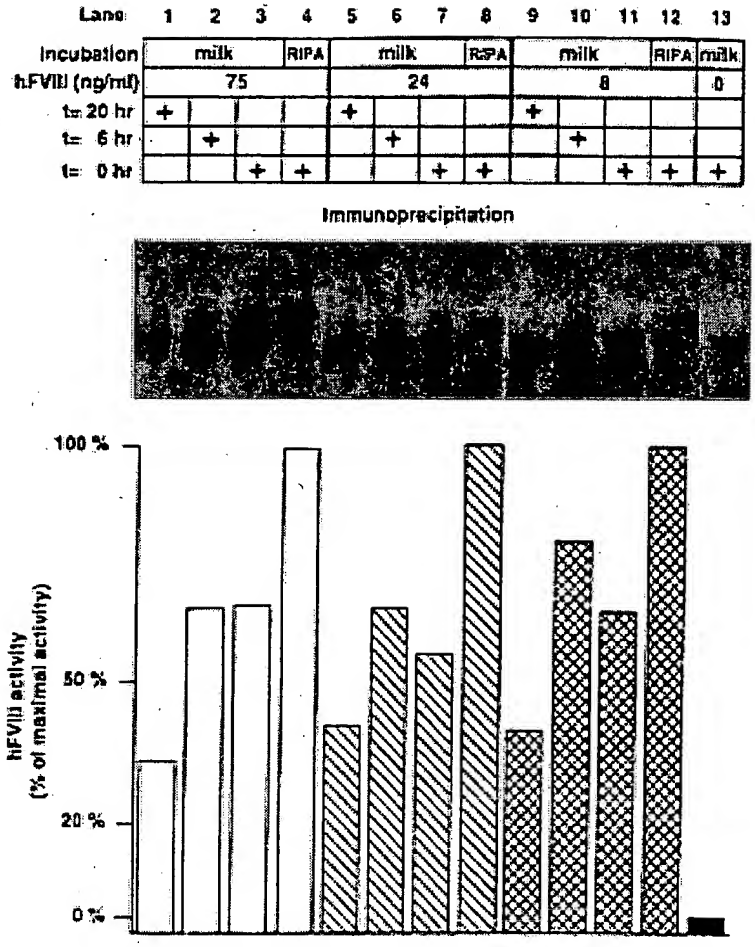

Figure 2b. Sequestration of rhFVIII^{std} in milk of a non-transgenic sheep. rhFVIII^{std} (either 8, or 24 or 75 ng/ml) were incubated in 500 µl undiluted milk samples for different times (0, 6, and 20 h) at 30°C as indicated in the top panel. At the indicated times, recovery of rhFVIII^{std} was determined by immunoprecipitation/immunoblotting (center panel), and in parallel by the clotting assay (low panel). Positive controls: the indicated amounts of rhFVIII^{std} in RIPA buffer without milk (see top panel) were immediately ($t = 0$) subjected to either immunoprecipitation/immunoblotting or to the clotting assay, and the recovered clotting activity de ned as = 100% (lanes 4, 8, 12). Negative control: milk without added rhFVIII^{std} (lane 13).

visualized by immunoprecipitable bands of higher mobility. The degradation of rhFVIII^{std} was partially inhibited by protease inhibitor PMSF.

When rhFVIII^{std} (120 ng/ml) was incubated in milk samples from non-transgenic ewes either freshly collected or after storage at −70°C at different temperatures, increasing losses of rhFVIII^{std} were detected with increasing temperature in both the immunoprecipitation/immunoblotting and clotting assays. Losses of rhFVIII^{std} were more apparent in the immunoblotting assay than in the clotting assay (Figure 2a), indicating that low rhFVIII^{std} levels that were not accessible to immunoprecipitation were readily identi ed in the clotting assay. The results demonstrated that rhFVIII^{std} in ovine milk was stable at 0°C, whereas increasing lability was observed following incubation at 28°C or 34°C. The stability of rhFVIII^{std} was not affected by storage of milk at −80°C.

Sequestration of rhFVIII^{std} in ovine milk

Following incubation of different concentrations of rhFVIII^{std} in undiluted non-transgenic ovine milk for different times, recovered rhFVIII^{std} was examined by

immunoblotting as well as by the clotting assay (Figure 2b). The results demonstrate a loss of rhFVIII^{std} over time in both detection systems. As expected, rhFVIII^{std} was clearly recovered as immunoprecipitate and visualized by immunoblotting, and identi ed by its biological activity when either 24 or 75 ng/ml rhFVIII^{std} had been incubated in milk. By contrast, no immunoprecipitable material was recovered following incubation of rhFVIII^{std} at the level of 8 ng/ml, although biological activity remained clearly detectable. This demonstrated that rhFVIII^{std} was sequestered at levels between 8 and 24 ng/ml in the RIPA buffer treated milk samples used here, rendering it cryptic and not accessible to immunoprecipitation, whereas biological activity was retained.

Discussion

In this report hFVIII cDNA constructs have been expressed in the mammary gland of a transgenic ewe for the rst time. In addition, the behaviour of the foreign protein in ovine milk was studied in considerable detail. The high pregnancy and lambing rates that were obtained in this study demonstrated that the gene constructs employed did not interfere with normal ovine development. The results indicate that the hFVIII cDNA containing transgene became stably integrated into the host genome, was transmitted to the offspring, expressed, the rhFVIII mRNA translated in the mammary gland, and the product secreted into the milk as biologically active rhFVIII protein. Mammary epithelial cells, which are the source of milk speci c proteins in the mammary gland, were shown to express the transgene. Exfoliated cells in ovine milk were viable after isolation as shown by their ability to attach to the plastic surface of tissue culture dishes and to actively multiply in primary culture (unpublished observation, 1998).

Although the presence of introns of the murine Mtl gene in the hFVIII cDNA construct presumably was instrumental for mediating expression, previous studies have shown that the use of introns in a plasmid-based hFVIII cDNA expression construct did not improve levels of the hFVIII protein (Lynch et al., 1993). Levels of biologically active rhFVIII protein in the milk of transgenic ewes 4010 and 603 attained 4–6 ng/ml at the end of the lactation period and are thus insuf cient for potential mass production of the blood clotting factor. Low ef ciency of expression of hFVIII cDNA constructs is a well-known problem in cell culture systems (Kaufman et al., 1988; Hoeben et al., 1995). The observed low expression rates of rhFVIII in the ovine mammary gland could be due to several factors including the structure of the transgene, the insertion site of the transgene in the genome (i.e. position effects) (Palmiter and Brinster, 1986), and to silencer-like sequences within the hFVIII encoding region (Lynch et al., 1993; Hoeben et al., 1995; Koeberl et al., 1995; Fallaux et al., 1996).

Three kilobases of the ß-lac 5′sequences were shown to be suf cient to support high levels of expression and longer sequences did not enhance expression levels (Shani et al., 1992). Nevertheless, it is possible that the 2.2 kb fragment of the ß-Lac promoter employed in our study lacked essential sequences required for directing expression of the hFVIII transgene exclusively to the mammary gland. This hypothesis is supported by the surprisingly high extent of ectopic expression of the hFVIII gene construct as observed in this study, con rming previous ndings in which similar and other gene constructs involving the ß-Lac or the whey acidic protein (WAP) promoter were employed in mice and sheep (Farini and Whitelaw, 1995; Wall et al., 1995; Espanion et al., 1997).

Silencer-like sequences were identi ed within a 1.2 kb stretch of hFVIII cDNA that were shown to act as dominant elements causing reduction of transcriptional activity of hFVIII cDNA constructs *in vitro* and ultimately resulting in a 30–100 fold reduction of expression ef ciency of hFVIII cDNA in comparison to vectors containing other cDNA sequences (Lynch et al., 1993). This occurred in an orientation and position dependent fashion (Koeberl et al., 1995). Thus, the observed low hFVIII cDNA expression rates were not due to reduced rates of transcriptional initiation rates but were presumably caused by a block of transcriptional elongation, in analogy to effects described for c-*myc* and CFTR genes (Koeberl et al., 1995). Furthermore, sequences localized within a 305 bp fragment of hFVIII cDNA were identi ed and shown to be involved in repressing transcription (Hoeben et al., 1995; Fallaux et al., 1996). This 305 bp fragment does not overlap with the region identi ed by Lynch et al. (1993), resembles the yeast autonomously replicating sequence (ARS) consensus and contains an element (nucleotides 1569–1600 of hFVIII cDNA) (Fallaux et al., 1996) that imposed repressing activity independent of its orientation in the transcription unit (Hoeben et al., 1995). Thus, the structure of the transgene involving hFVIII cDNA including the silencer-like elements in the hFVIII coding region as

well as the 2.2 kb ß-Lac promoter fragment employed here, could in part be responsible for the low expression levels of hFVIII protein production achieved in recombinant CHO cells or broblasts (Kaufman et al., 1988; Hoeben et al., 1990) and in the mammary gland of the transgenic ewes reported here.

It is known that the mammary gland contains the enzymatic machinery required for the correct synthesis and processing of complex proteins that includes extensive post-translational modi cations (Hennighausen et al., 1990; Wilmut et al., 1990). By directing the synthesis of the desired protein into the mammary gland through appropriate gene constructs by means of a mammary gland speci c promoter element it has been possible to express various genes in transgenic farm animals. Regulatory sequences of the rodent WAP, ovine ß-lactoglobulin, bovine α-lactalbumin and bovine and caprine casein genes have been employed to target expression of different genes to the lactating mammary gland in laboratory and farm animals, including tissue plasminogen activator (tPA), human protein C, human antihemophilic factor IX and human α1-antitrypsin. Expression levels have been variable and usually were approximately 1–2 μg/ml, but high expression levels (63 μg/ml) have been reported (Gordon et al., 1987; Simons et al., 1987; Pittius et al., 1988; Clark et al., 1989; Archibald et al., 1990; Burdon et al., 1991; Ebert et al., 1991; Wall et al., 1991; Wright et al., 1991; Velander et al., 1992). Recently, a transgenic pig carrying the cDNA of the human FVIII gene under the transcriptional control of the regulatory sequences of the murine WAP gene was shown to secrete up to 2.7 μg/ml in milk, which is tenfold higher than in normal plasma (Paleyanda et al., 1997). Speculatively, the porcine mammary could be a better suited production system for rhFVIII than the ovine mammary gland. But also differences in the construct design or integration site could have contributed to different expression levels in the present study and that from Paleyanda et al. (1997).

Although the behaviour of exogenous rhFVIII^{std} may not be fully representative for that of endogenously produced rhFVIII in milk of the transgenic ewes described in the present study, the results highlight the problems that are encountered when attempts are made to express complex, large genes in the mammary gland of transgenic livestock. The results of this study indicate that conventional constructs of the type ß-Lac/hFVIII-MtI may not necessarily be useful for directing expression of rhFVIII to the ovine mammary gland to produce large amounts of the desired protein. Although it is well-known that hFVIII protein levels in blood are low (100–200 ng/ml) when compared to clotting factor IX (4–8 μg/ml) (Fallaux et al., 1995), and hFVIII mRNA in animals accounts to only 0.001% of liver poly(A)$^+$ RNA (Wion et al., 1985), it is possible that the ef ciency of transgene expression can be improved by employing novel constructs containing genomic DNA encoding hFVIII. A promising approach could be to employ hFVIII genomic sequences cloned into arti cial chromosome vectors such as YACs (Naylor et al., 1993). These vectors permit the transfer of large (up to 1000 kb) transgenes into zygotes and their subsequent expression in the desired target organ (Brownstein et al., 1989; Schedl et al., 1993). A transgenic ewe expressing high levels of rhFVIII will be of great value for the mass production of biologically active rhFVIII. Finally, procedures for an ef cient puri cation of biologically active rhFVIII from ovine milk are yet to be developed.

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